

Pulmonary but not subcutaneous vaccination confers protection to TB susceptible mice by an IL17-dependent mechanism

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ABSTRACT

Some of the most promising novel tuberculosis (TB) vaccine strategies currently under development are based on respiratory vaccination, mimicking the natural route of infection. In this work, we have compared pulmonary and subcutaneous BCG immunization in the TB susceptible DBA/2 mouse strain, a model in which parenteral BCG does not protect. Our data show that intranasal but not subcutaneous BCG confers robust protection against pulmonary TB challenge. In addition, our results indicate that pulmonary vaccination triggers a TB-specific mucosal immune response orchestrated by IL17A. Thus, IL17A neutralization *in vivo* reduces protection, as well as it abrogates TB-specific IgA secretion to respiratory airways and lung expression of pIgR induced following intranasal vaccination. Altogether, our results demonstrate that pulmonary BCG vaccination can overcome lack of protection observed when BCG is given by parenteral route, suggesting that respiratory TB vaccines could have an advantage in TB endemic countries, where intradermal BCG results inefficient against pulmonary TB.

BACKGROUND

Tuberculosis (TB) disease causes one and a half million deaths per year, and is one of the leading infectious diseases affecting mainly developing and underdeveloped countries. The rising spread of multidrug resistant strains with the increasing globalization makes TB an alarming global health problem [1]. Therefore, there is an urgent need for new effective TB vaccines.

The only vaccine against TB in use today, the Bacille Calmette-Guerin (BCG), is a live attenuated strain of *Mycobacterium bovis*. BCG was initially designed as an oral vaccine, but since the 1970s intradermal administration at birth was established worldwide. Nowadays, BCG is considered effective in reducing the rate of severe forms of TB (meningitis and miliary TB) in children, but is inconsistent in preventing spread of pulmonary TB responsible of disease transmission [2].

Vaccination through the natural route of infection represents an attractive approach in vaccinology for priming the natural host immunity. In the case of TB, pulmonary mucosal tissue is the primary site for establishment of infection.

Nevertheless, it is unclear which are the primary defence mechanisms of the mucosal immune response triggered during early TB infection. IL17A has emerged as a key molecule in the induction and maintenance of mucosal immunity [3-5].

Although this cytokine was initially described to participate in the elimination of extracellular pathogens and fungi [6, 7] due to its neutrophil recruitment capacity [8], it can also contribute to protection against intracellular bacteria [9, 10].

Results obtained with IL17A-knockout mice reveal no greater sensitivity to *Mycobacterium tuberculosis* (MTB) than wild-type mice in short-term TB challenge experiments [11]. However, a recent study has shown that this result depends on the virulence level of the MTB strain used for infection [12]. IL17A is known to

orchestrate the optimal organization of granuloma during TB infection in mice [13, 14], which could explain the higher mortality rate of IL17RA-deficient mice in long-term TB infections [15]. Regarding the role of mucosal immunoglobulins (Ig) during mycobacterial infection, IgA-deficient mice are more sensitive to pulmonary challenge with BCG [16], suggesting a role for IgAs in mycobacterial infections. Mice knockout for the polymeric Ig receptor (pIgR), a protein expressed in the mucosal epithelium whose function is to translocate IgAs from lamina propria to the gut lumen or lung airways [17, 18], show greater susceptibility to TB challenge [19].

Depending on their sensitivity to TB infection, mouse strains have been classified into susceptible and non-susceptible [20]. Besides their different susceptibility profile to TB challenge, these strains also differ in the protective response following parenteral BCG vaccination. Whereas resistant strains C57/BL6 and BALB/c, the most used mouse models for TB vaccine comparison, show good vaccine-induced protection, parenteral immunization with BCG does not confer protective efficacy in susceptible strains CBA/J or DBA/2 [21, 22].

In this work, we carried out a comparative study of intranasal and subcutaneous BCG vaccination in the TB susceptible mouse strain DBA/2. Our data demonstrate that pulmonary BCG delivery overcomes lack of protection observed after subcutaneous vaccination in a process mediated by IL17A.

MATERIALS AND METHODS

Bacteria

Mycobacterial strains were grown at 37°C in Middlebrook 7H9 broth (BD Biosciences) supplemented with ADC (BD Biosciences) and 0.05% (v/v) Tween-80, or on solid Middlebrook 7H11 (BD Biosciences) supplemented with ADC. Bacterial suspensions for vaccination or infection were prepared in PBS from glycerol stocks previously quantified.

Mice

All mice were kept under controlled conditions and observed for any sign of disease. Experimental work was conducted in agreement with European and national directives for protection of experimental animals and with approval from the competent local ethics committees.

For protection studies, groups of eight week-old female C57/BL6 or DBA/2JRj mice (Janvier Biolabs) were vaccinated subcutaneously (100 µl) or intranasally (40 µl) with 10⁶ CFU of BCG Danish 1331 in PBS. Eight weeks post-vaccination, mice were intranasally challenged with 100 CFU (low-dose challenge) or 1000 CFU (high-dose challenge) of MTB H37Rv in 40 µl of PBS. Bacterial load from homogenized lungs and spleen was determined four weeks post-challenge by plating on solid medium. For survival experiments, disease-associated symptoms (including weight, aspect and individual/social behaviour) were monitored weekly, and mice were humanely euthanized according to pre-established endpoint criteria. Histological studies were performed according to a previous work [23].

For immunogenicity studies, eight week-old female DBA/2JRj mice were vaccinated subcutaneously or intranasally with 10⁶ CFU of BCG Danish 1331 in

PBS. At the indicated time points, animals were euthanized and splenocytes and lung cells collected and stimulated with Purified Protein Derivative (PPD) (Statens Serum Institute, SSI) 5 µg/ml overnight for intracellular staining (ICS), or during 48 hours for supernatants collection and cytokine detection by ELISA. For bronchoalveolar lavage (BAL) collection, trachea was cannulated and BAL was performed with 0.8 ml of ice-cold PBS. Supernatant was separated from cells by centrifugation and frozen at -80°C for further IgA detection analysis.

IL17A neutralization in vivo

IL17A neutralization was performed as described previously [24]. Briefly, mice were inoculated intraperitoneally with 500 µg of antiIL17A clone 17F3 (BioXcell) or isotype control in 100 µl of PBS the day before vaccination and twice a week thereafter until experiment completion.

Flow cytometry

For intracellular staining (ICS), GolgiPlug (BD Biosciences) was added to cells during the last six hours of incubation with PPD. Then, cells were fixed and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) following manufacturer instructions. Cells were stained with antiIL17A-APC.Cy7 and antiIFN γ -APC (BD Biosciences). For surface staining, cells were labelled with antiCD4-FITC, antiCD8-PE, antiLy6G-Pacific Blue, CD11b-PE (BD Biosciences) or CD11c-FITC (Miltenyi Biotec) diluted in culture medium with 10% FCS. Cells were acquired with a Gallios Flow Cytometer (Beckman).

ELISA

Cytokine concentration in supernatants was determined by using mouse IL17A or IFN γ specific ELISA kits (MabTECH).

For IgAs determination in BAL, maxisorp ELISA plates (NUNC) were coated with BAL (for total IgA) or 10 µg/ml of H37Rv sonicate (for MTB-specific IgA) and incubated overnight at 4°C. After a washing step with PBS-Tween20 0.05% (v/v) buffer, plate was blocked with Bovine Serum Albumin 1% (w/v) in washing buffer for 1 hour at 37°C. Then, MTB sonicate-coated plates were incubated with 200 µl of BAL during 90 minutes at 37°C. Following washing, plates were incubated for 1 hour at 37°C with Horseradish Peroxidase (HRP)-conjugated goat anti-mouse IgA diluted 1:10000 (Sigma). Finally, enzyme-substrate reaction was developed using 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma) as substrate, and reaction was stopped with H₂SO₄ 0.1N. Standard curve to calculate IgA concentration was performed using a mouse reference serum (Bethyl technologies). Optical density was measured at 450 nm.

Western-Blot

Lung protein lysates were prepared by adding 100 µl of RIPA buffer (2x concentrated) to 100 µl of homogenised lung followed by boiling at 100°C for 1 hour. Then, supernatant was separated by centrifugation at 14000 x g for 15 minutes, and protein concentration measured with the QuantiPro BCA Assay kit (Sigma). Ten micrograms of protein per well were loaded and separated by SDS-PAGE. Immunodetection was carried out using a polyclonal goat anti-pIgR (R&D systems). Then, membranes were incubated with HRP-conjugated anti goat IgG secondary antibody (Sigma) and developed with ECL Plus Western Blotting System (GE HealthCare). Band intensities were calculated with the ImageJ software.

Statistical Analysis

GraphPrism software was used for statistical analysis. For experiments with two experimental groups, unpaired t-student test was used. When three or more

groups were compared, One-Way ANOVA analysis with Bonferroni post-test was performed. Differences were considered significant at $p < 0.05$.

RESULTS

Intranasal but not subcutaneous BCG vaccination confers protection to DBA/2 against *Mycobacterium tuberculosis* challenge

Previous data indicated that subcutaneous vaccination with BCG does not protect against TB in the susceptible mouse strain DBA/2 [21, 22]. To confirm these data under our experimental conditions, groups of C57/BL6 and DBA/2 mice were subcutaneously vaccinated with BCG Danish and after intranasal challenge with a low dose of H37Rv, we measured bacterial burden in lungs four weeks later. Vaccinated C57/BL6 animals showed one-log reduction as compared to non-vaccinated group (**Figure 1A**), whereas no difference was observed between both vaccinated and unvaccinated DBA/2 mice (**Figure 1B**).

Recently, we reported that pulmonary BCG is more effective than subcutaneous vaccination in C57/BL6 mice [25]. As a result we considered extending these studies to DBA/2 mice in a short-term protection design to explore whether vaccination by the natural route of infection could protect against TB in a model where parenteral BCG immunization fails. After low dose H37Rv challenge, intranasal BCG reduced bacterial burden in lungs by about two logs (**Figure 2A**) and approximately by one log after high dose challenge, as compared to unvaccinated or subcutaneous group (**Figure 2B**). Comparable results were obtained in spleen.

To test whether this considerable bacterial load reduction by intranasal vaccination could be translated into increased survival and reduced disease, we

conducted a long-term survival study comparing subcutaneous and intranasal vaccination following high dose H37Rv challenge. Concordant with lung-bacterial load data in the short-term protection study, intranasal vaccination significantly increased mouse survival with a median of around four months post-challenge, whereas median survival of subcutaneous and non-vaccinated animals was about one month (**Figure 2C**). Altogether, these results indicate that intranasal BCG delivery overcomes lack of protective efficacy by subcutaneous route in DBA/2 mice.

Additionally, we explored whether BCG intranasal immunization could induce lung damage in this mouse model. Tissue damage caused by exacerbated inflammation is probably the main concern for the use of live TB vaccines by the pulmonary immunization route in humans. Our results revealed the presence of some histopathological findings in the lungs from animals vaccinated intranasally. We detected the presence of discrete inflammatory aggregates in a small proportion of lung tissue and mainly surrounding blood vessels. These findings were not found when animals were vaccinated subcutaneously (**Supplementary Figure 1**).

Intranasal BCG vaccination induces IL17A production

Th17 response has been shown to correlate with vaccine-induced protection in preclinical animal models [26]. Given the protective efficacy obtained in the DBA/2 mice after switching to the pulmonary route of vaccination, we analyzed T-helper 17 induction in lungs induced by vaccination and before challenge. Higher frequency (**Figure 3A**) of PPD-specific IL17A-producing CD4⁺ T cells was observed in the mouse group vaccinated intranasally as compared to the parenteral or non-vaccinated groups. IL17A production measured by ELISA confirmed intracellular staining results (**Figure 3B**). Increased IL17A production

was also observed in spleen from the intranasal group (**Figure 3C**), suggesting that in this model the pulmonary route of vaccination is immunogenic not only at local level in lungs, but also systemically. Concurring with these data, we observed a similar IFN γ induction profile in lungs (**Figure 4A, 4B**) and spleen (**Figure 4C**), as shown with IL17A.

BCG-conferred protection correlates with pulmonary IL17A induction post-challenge

To investigate whether Th17 response induced by pulmonary vaccination in DBA/2 mice correlated with protection in lungs, we measured IL17A production in parallel with bacterial load reduction four weeks post-low-dose H37Rv challenge. Our results indicated that induction of IL17A-secreting CD4⁺ T cells after challenge was restricted to the intranasal BCG-vaccinated group (**Figure 5A left panel**).

Linear regression comparing percentage of IL17A⁺ CD4⁺ T cells and bacterial load in lungs (**Figure 5A right panel**) indicated a significant correlation between reduction in lung bacterial burden and Th17 response induction, providing evidence that IL17A could mediate BCG-induced protective efficacy by the intranasal route.

Conversely, we observed no differences in percentage of IFN γ -producing CD4⁺ T cells post-challenge between vaccinated and non-vaccinated animals (**Figure 5B left panel**). Indeed, comparing Th1 induction and lung bacterial load we found a weak but significant positive correlation between cytokine production and CFU counts (**Figure 5B right panel**).

IL17A contributes to protection conferred by intranasal vaccination

To study the contribution of IL17A production to protection conferred by pulmonary vaccine delivery, we neutralized IL17A in intranasally vaccinated

animals. As shown in **Figure 6A**, IL17A blocking with a specific antibody partially diminished BCG efficacy conferred by intranasal vaccination, confirming a role of this cytokine in protection. IL17A neutralization was confirmed by the substantial cytokine reduction produced in lungs after antibody treatment (**Figure 6B**).

Residual IL17A production found in the antibody-treated group could explain the remaining protection still detected after IL17A neutralization, although IL17A-independent factors cannot be excluded. In contrast to IL17A, IFN γ lung production was unaffected in the absence of IL17A (**Figure 6C**).

We studied whether neutrophil recruitment in lungs was affected by IL17A neutralization, as this process had been previously related with Th17 response [13]. Intranasal BCG vaccination caused neutrophil infiltration in lungs.

Interestingly, IL17A inhibition led to a drop of the percentage as well as the number of neutrophils. However, total cell infiltration occurred in the two intranasal groups regardless of IL17A neutralization, suggesting that this process is mediated by IL17A-independent inflammatory events triggered following pulmonary vaccination with BCG (**Figure 6D**).

IL17A mediates MTB-specific IgA secretion to lung airways

IgA represents one of the main defence barriers of the mucosal immune system. Therefore, we analysed BAL samples to determine the presence of total and MTB-specific IgA in the lung airways induced eight weeks post-vaccination. Our results showed an increment of total and MTB-specific IgA in BAL from intranasally BCG vaccinated mice (**Figure 7A**). Interestingly, following IL17A-neutralization only presence of MTB-specific IgA in BAL was significantly diminished, whereas total IgA was unaffected by antibody treatment. Provided that pIgR is the receptor molecule responsible for the transport of IgA across lung epithelium, we analysed

whether pIgR expression would be altered upon vaccination. Our data indicated an increase of pIgR protein expression in the lungs of intranasally BCG vaccinated mice (**Figure 7B**), which correlated with higher IgA concentration in BAL found in these groups. Moreover, our results suggested that pIgR upregulation depended on IL17A production, as pIgR protein expression levels were significantly lower in the anti-IL17A-treated group as compared to the intranasally immunized untreated group. These results suggest that translocation of MTB-specific IgA to respiratory airways and increased pIgR expression levels correlates with IL17A-mediated protection upon pulmonary BCG immunization in DBA/2 mice.

DISCUSSION

Pulmonary vaccination with BCG has been previously reported to improve protection when compared with parenteral (subcutaneous or intradermal) inoculation [25, 27-29]. Nevertheless, experiments showing these results have been usually performed in TB resistant mouse strains (C57BL/6 and BALB/c) in which parenteral BCG is considered to be protective. In this work, we demonstrate for the first time to our knowledge that a change in the route of vaccination from parenteral to pulmonary can overcome the absence of protection observed in a model where parenteral BCG does not protect, as it occurs in the TB susceptible DBA/2 mouse strain. Extrapolation of these data to TB endemic countries, where protection conferred by intradermal BCG is limited, suggests that pulmonary BCG administration could have an impact against respiratory TB. Nonetheless, our data suggest caution when using BCG by the respiratory route due to the inherent safety risks associated with dissemination and unspecific inflammation. Intranasal BCG vaccination of DBA/2 mice was accompanied by lung inflammatory aggregate

formation and neutrophil infiltration, events usually associated with acute inflammation and tissue damage (**Supplementary Figure 1** and **Figure 6C**, respectively). In agreement with these data, a previous work described that the number of pulmonary BCG immunizations is also critical to induce an exacerbated damaging inflammatory response [30]. In addition, considerable number of BCG bacilli was found in lungs two months post-vaccination (**Supplementary Figure 2**), which could entail some safety concerns in immunocompromised individuals. Nevertheless, it is important to remark that BCG administration by the aerosol route has been already tested in humans as lung cancer immunotherapy approach, showing satisfactory safety results [31], indicating that inflammatory events caused by pulmonary BCG vaccination are most likely transient and are not harmful at long-term.

Some authors have described a reduced expression of adhesion molecules on the surface of circulating lymphocytes from TB susceptible mice including DBA/2, which correlates with a poor presence of lymphocytes in lungs after TB infection [21, 32]. Therefore, a plausible explanation as to why subcutaneous BCG immunization does not protect in DBA/2 mice could be that vaccine-induced systemic immune response is unable to efficiently reach the site of infection. Intranasal vaccination could eventually overcome these deficiencies leading to antigen presentation directly in the lung draining lymph nodes and the subsequent local immune response establishment prior to pathogen encounter. Supporting this hypothesis, we have shown that intranasal but not subcutaneous BCG vaccination triggers robust Th1 and Th17 responses in lungs of DBA/2 mice. Our data strongly suggest that Th17 response induction is particularly important for protection against TB conferred by pulmonary BCG vaccination, which is in

accordance with previous observations made with other TB vaccines [11, 33]. A recent report in TB-infected macaques has demonstrated higher presence of IL17-producing cells in sterile granulomas as compared to non-sterile ones [34]. The induction of Th17 response by intranasal but not subcutaneous vaccination could be related with the local inflammatory ambient caused by the presence of BCG in the lungs, which would likely provide pro-inflammatory cytokines crucial for the differentiation of Th17 cells [35]. Contribution of other IL17-expressing cellular subsets, as gamma delta T cells or neutrophils [36], should not be discarded as potential contributors of vaccine-induced IL17 production, in addition to Th17 cells. Our data indicate that IL17A neutralization did not totally abrogate BCG intranasally-induced protection, suggesting that other factors may possibly be implicated. Further work is needed to understand whether IL17A-independent pathways (i.e. IFN γ production, other IL17 family cytokines) are implicated in protective efficacy conferred by pulmonary vaccination.

A previous work by Khader *et al.* found that vaccination-induced IL17A enhances IFN γ -producing CD4⁺ T cell recruitment to the lungs, mainly at very early timepoints post challenge [26]. Our data are in apparent discordance with these results as we found similar IFN γ production induced by intranasal vaccination independently of IL17A (**Figure 6C**). However, at the time when we measured cytokine production (at two months post-vaccination) IFN γ -producing cells could likely have already been recruited to the lungs independently of IL17A. Indeed, these authors detected IFN γ production in lungs in the absence of IL17A at longer time points post-challenge.

Immunoglobulins can be translocated to respiratory tract passively via blood leakages, or actively crossing lung epithelium via pIgR interaction, which is specific

for IgA and IgM subtypes [37]. IL17A dependence on both MTB-specific IgA translocation and pIgR upregulation suggests that specific IgAs are produced locally in lung mucosal tissue, where mycobacterial antigens are abundant after pulmonary vaccination, and translocated to respiratory airways exclusively via pIgR. Conversely, IL17A-independent translocation of total unspecific IgAs, which occurs in the absence of pIgR upregulation, likely indicates that in this case it arises from blood leakages. Previous works have described that IL17A contributes to IgA secretion in gut and airways as well as pIgR expression in lung epithelium [17, 38]. However, the link between IL17A with pIgR expression and IgA secretion in the context of mucosal TB vaccines was unreported. Both pIgR and IgA knockout mice have shown to be defective in controlling mycobacterial replication [16, 19], which could suggest a role of IgAs in protection. Nevertheless, our data do not discern whether IgA-induction is a causative factor that contributes to intranasally-induced BCG protection.

Even though different works already showed some decades ago the advantages of pulmonary vaccination to protect against TB, no significant attempts to translate these results to clinic have been made during the last 20 years. However, and probably influenced by the failure of intradermal MVA85A vaccine to improve BCG protection in clinic [39], in the last few years a new awareness among the TB vaccine scientific community has emerged suggesting that elimination of TB will not be achieved only with new vaccine candidates, and exploration of novel approaches including new routes of immunization is necessary. In this regard, a rising and renewed interest in mucosal immunization has appeared among vaccine developers, which is demonstrated by the recently published first clinical trial to test the MVA85A subunit vaccine delivered by aerosol route [40]. Therefore,

elucidation of protective mechanisms triggered by mucosal immunization might be highly valuable in the near future for the rational design of new pulmonary vaccines, as well as for the identification of specific biomarkers for these vaccines that could predict protection against TB.

Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. BCG subcutaneous vaccination protects C57/BL6 but not DBA/2

mice against a pulmonary challenge.

Groups of six C57/BL6 (A) or DBA/2 (B) mice were vaccinated by the subcutaneous route with BCG Danish vaccine 10^6 CFU. At two months post-vaccination, mice were inoculated intranasally with a low dose H37Rv challenge (100 CFU), and one month later lung bacterial burden was determined. A representative experiment of two independent is shown. Data in the graphs are represented as mean \pm SEM. Unpaired t-student test was performed to calculate statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2. BCG intranasal vaccination protects DBA/2 against a pulmonary

challenge.

Groups of six DBA/2 mice were vaccinated by the subcutaneous (BCG sc), intranasal (BCG in) route, or non-vaccinated (NV) with BCG Danish vaccine 10^6 CFU. At two months post-vaccination, mice were inoculated intranasally with a low dose (100 CFU) (A) or high dose (1000 CFU) (B) H37Rv challenge, and one month later bacterial burden in lungs and spleen was determined. A representative experiment of two independent is shown. Data in the graphs are represented as mean \pm SEM. One-way ANOVA test with Bonferroni post analysis was performed to calculate statistical significance. (C) For survival experiments, groups of eight animals were vaccinated and challenged with a high dose H37Rv challenge two months post-vaccination. Animal survival was determined according to pre-established endpoint criteria approved by ethical committee. Data from one experiment are represented in a Kaplan-Meier survival curve and statistical significance calculated by a Logrank test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3. BCG intranasal vaccination induces a higher Th17 response prior to

challenge.

Groups of six DBA/2 mice were vaccinated by the subcutaneous (BCG

sc) or intranasal (BCG in) route, or non-vaccinated (NV) with BCG Danish vaccine 10^6 CFU. At two months post-vaccination, a cellular suspension from harvested lungs (**A, B**) or spleen (**C**) was obtained. Cells were stimulated with PPD as described in materials and methods section. CD4+IL17A+ cells frequency in lungs was determined by flow cytometry (**A**). IL17A concentration in lung (**B**) or spleen (**C**) cell supernatants was measured by ELISA. Pooled data from two independent experiments are shown. Data in the graphs are represented as mean \pm SEM. One-way ANOVA test with Bonferroni post analysis was performed to calculate statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 4. BCG intranasal vaccination induces a higher Th1 response prior to challenge. Groups of six DBA/2 mice were vaccinated by the subcutaneous (BCG sc) or intranasal (BCG in) route, or non-vaccinated (NV) with BCG Danish vaccine 10^6 CFU. At two months post-vaccination, a cellular suspension from harvested lungs (**A, B**) or spleen (**C**) was obtained. Cells were stimulated with PPD as described in materials and methods section. CD4+IFN γ + cells frequency in lungs was determined by flow cytometry (**A**). IFN γ concentration in lung (**B**) or spleen (**C**) cell supernatants was measured by ELISA. Pooled data from two independent experiments are shown. Data in the graphs are represented as mean \pm SEM. One-way ANOVA test with Bonferroni post analysis was performed to calculate statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5. Th17 response induced post challenge correlates with a lower bacterial load in lungs. Groups of six DBA/2 mice were vaccinated by the subcutaneous (BCG sc) or intranasal (BCG in) route, or non-vaccinated (NV) with BCG Danish vaccine 10^6 CFU. A control group of non-vaccinated, non-infected mice was also included (NV/NI). At two months post-vaccination, mice were challenged

intranasally with a low H37Rv dose (100 CFU), and one month later animals were euthanized. Right and left lungs from the same animal were used to determine bacterial load and IL17A- (A) or IFN γ - (B) producing CD4+ cells, respectively. Data in left panels correspond to percentage of cytokine-producing cells measured by flow cytometry, and are represented as mean \pm SEM. One-way ANOVA test with Bonferroni post analysis was performed to calculate statistical significance. *

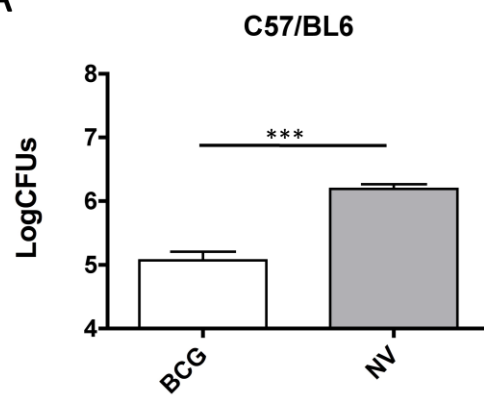
$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data from lung CFU and cytokine- producing CD4+ cells obtained for each mouse were graphically represented (right panels). Linear regression was calculated and the p-value obtained in each case is shown in the graph. Pooled data from two independent experiments are shown in the figure.

Figure 6. IL17A neutralization *in vivo* impairs protection conferred by BCG

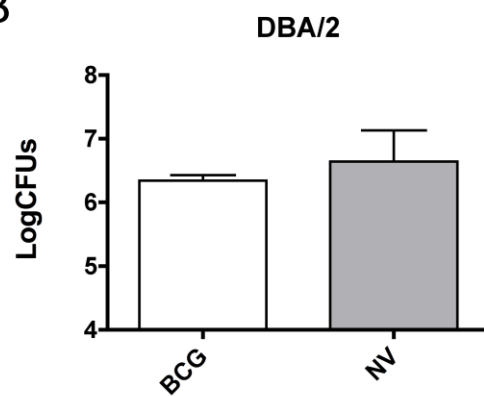
intranasal vaccination. Groups of six DBA/2 mice were vaccinated by the subcutaneous (BCG sc) or intranasal (BCG in) route, or non-vaccinated (NV) with BCG Danish vaccine 10^6 CFU. Intranasal vaccinated mice were treated twice a week with 500 μ g of antiIL17A or isotype control inoculated intraperitoneally. (A) At two months post-vaccination, mice were challenged intranasally with a low H37Rv dose (100 CFU), and one month later lung bacterial burden was determined. (B, C) A group of mice was euthanized prior to challenge and IL17A (B) or IFN γ (C) concentration measured in lung cell culture supernatants after PPD stimulation. D, total cell number (left panel), % of neutrophils (defined as Ly6G+CD11b+CD11c-cells) (mid panel), and number of neutrophils (right panel) were determined in lungs prior to challenge. A representative experiment of two independent is shown. Data in the graphs are represented as mean \pm SEM. One-way ANOVA test with Bonferroni post analysis was performed to calculate statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

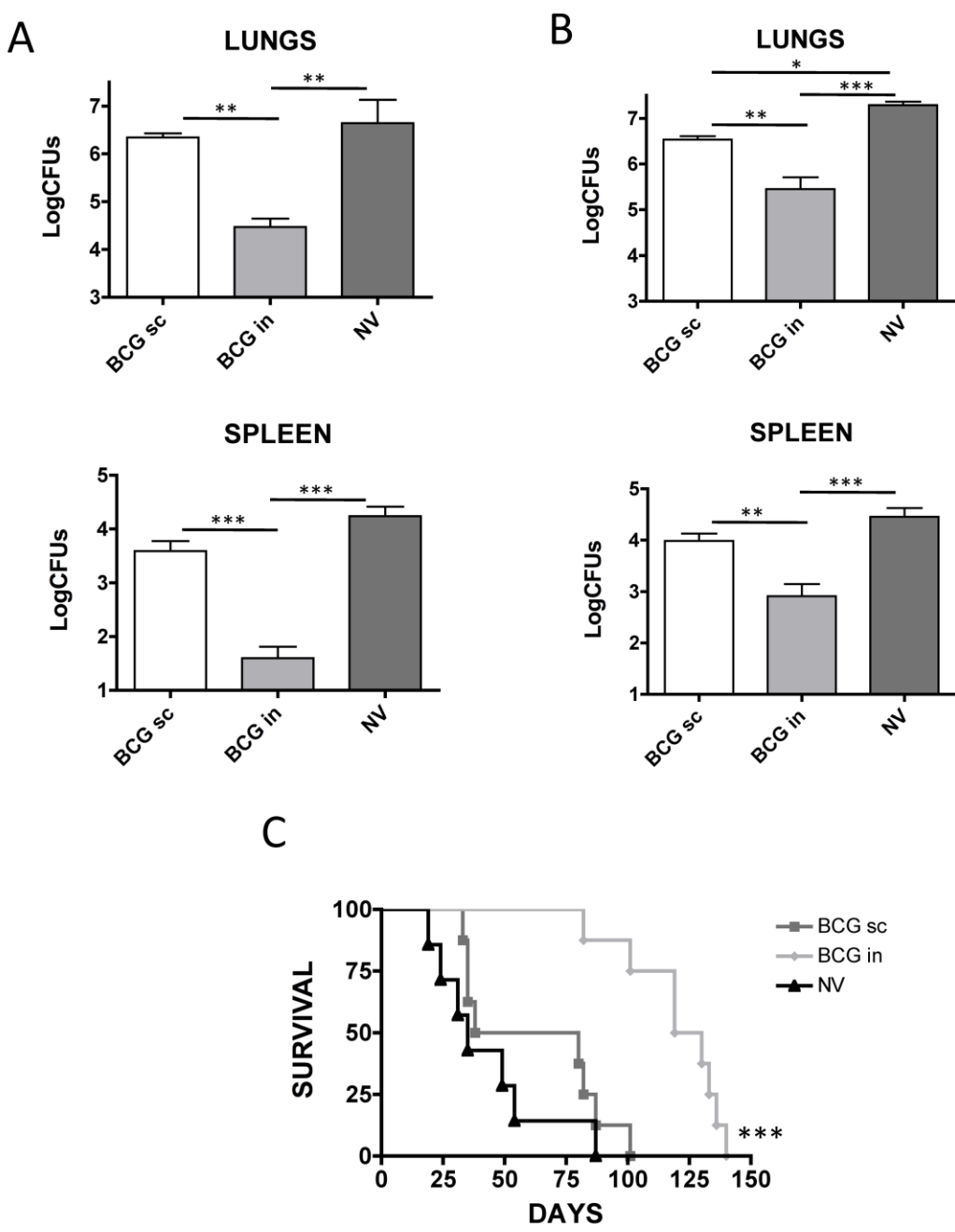
Figure 7. BCG intranasal vaccination induces secretion of MTB-specific IgAs and pIgR expression in lungs in an IL17A-dependent manner. Groups of six DBA/2 mice were vaccinated by the subcutaneous (BCG sc) or intranasal (BCG in) route, or non-vaccinated (NV) with BCG Danish vaccine 10^6 CFU. Intranasal vaccinated mice were treated twice a week with 500 μ g of antiIL17A or isotype inoculated intraperitoneally. **(A)** At two months post-vaccination, animals were euthanized and total and MTB-specific IgA levels measured in BAL as described in materials and methods section. **(B)** Expression of pIgR was measured in 10 μ g of lung lysates by western-blotting. Representative data from two mice per group are shown in the western-blot. Pooled data from two independent experiments are shown in the graphs. Data in the graphs are represented as mean \pm SEM. One-way ANOVA tests with Bonferroni post analysis were performed to calculate statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

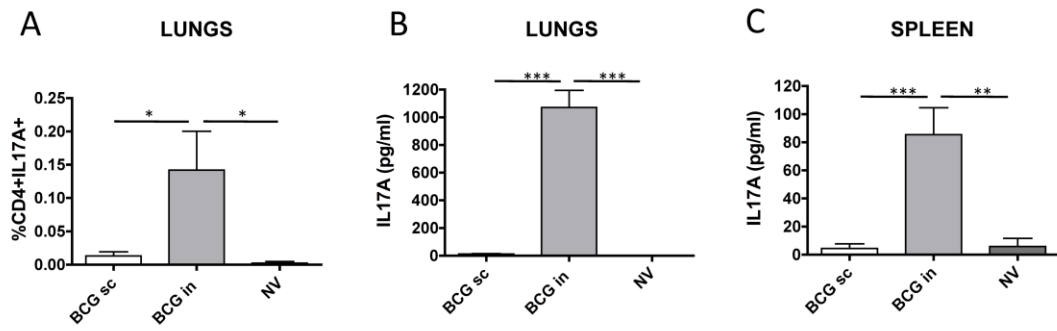
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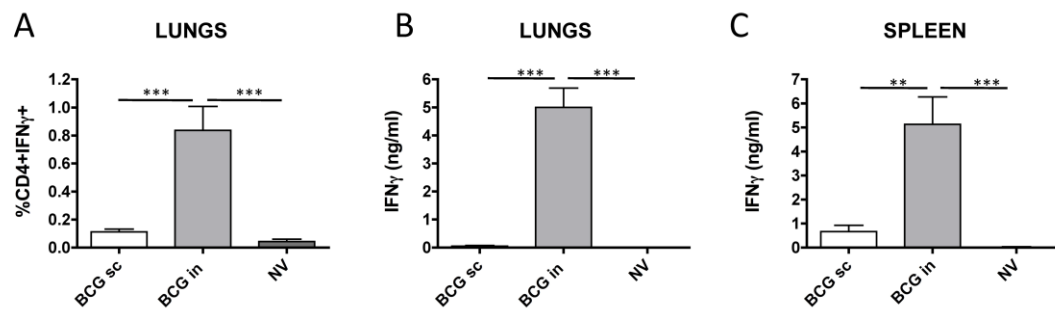


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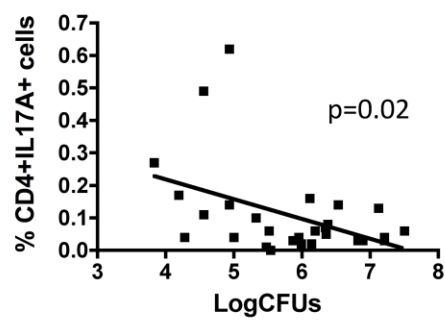
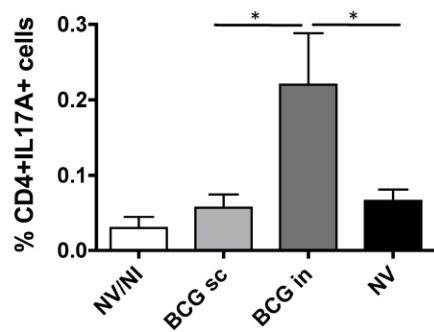








A



B

